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(54) Title: GENE CODING FOR THE E1 ENDOGLUCANASE

(57) Abstract

The gene encoding *Acidothermus cellulolyticus* E1 endoglucanase is cloned and expressed in heterologous microorganisms. A new modified E1 endoglucanase enzyme is produced along with variants of the gene and enzyme. The E1 endoglucanase is useful for hydrolyzing cellulose to sugars for simultaneous or later fermentation into alcohol.

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Gene Coding for the E1 Endoglucanase

This application is a continuation-in-part of serial number 08/125,115 filed September 21, 1993, pending, which is a continuation-in-part of 07/826,089 filed January 27, 1992, now U.S. Patent 5,275,944, which was a continuation-in-part of serial number 412,434 filed September 26, 1989 now U.S. Patent 5,110,735.

The United States Government has rights in this invention under Contract No. DE-AC36-83CH10093 between the United States Department of Energy and the National Renewable Energy Laboratory, a Division of the Midwest Research Institute.

Technical Field

The invention relates to genes encoding *Acidothermus cellulolyticus* E1 endoglucanase, recombinant microorganisms containing the gene and their use to express the gene to produce the enzyme or to degrade cellulose.

Background Art

The fermentable fractions of biomass include cellulose (β-1,4-linked glucose) and hemicellulose. Cellulose consists of long, covalently bonded insoluble chains of glucose which are resistant to depolymerization. Hemicellulose is a heterogeneous fraction of biomass that is composed of xylose and minor five- and six-carbon sugars. Although it is an abundant biopolymer, cellulose is highly crystalline, insoluble in water, and highly resistant to depolymerization. The complete enzymatic degradation of cellulose to glucose, probably the most desirable fermentation feedstock, may be accomplished by the synergistic action of three distinct class of enzymes. The first class, the "endo-1,4-β-glucanases" or 1,4-β-D-glucan 4-glucanohydrolases (EC 3.2.1.4), acts at random on soluble and insoluble 1,4-β-glucan substrates to brake the chains and are commonly measured by the detection of reducing groups released from carboxymethylcellulose (CMC). The second class, the "exo-1,4-β-glucosidases", includes both the 1,4-β-D-glucan glucohydrolases (EC 3.2.1.74), and 1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91). These enzymes respectively liberate D-glucose from 1,4-β-D-glucans and hydrolyze D-cellobiose slowly, and liberate D-cellobiose from 1,4-β-glucans. The third class, the "β-D-glucosidases" or β-D-glucoside glucohydrolases (EC 3.2.1.21), act to release D-glucose units from soluble cellodextrins and an array of glycosides.

The development of an economic process for the conversion of low-value biomass to ethanol via fermentation requires the optimization of several key steps, especially that of cellulase production. Practical utilization of cellulose by hydrolysis with cellulase to produce glucose requires large amounts of cellulase to fully depolymerize cellulose. For example, about one kilogram cellulase preparation may be used for every fifty kilograms of cellulose. Economical production of cellulase is also compounded by the relatively slow growth rates of cellulase producing fungi and the long times required for cellulase induction. Therefore, improvements in or alternative cellulase production systems capable of greater productivities of cellulase activity than may be possible from natural fungi would significantly reduce the cost of cellulose hydrolysis and make the large-scale bioconversion of cellulosic biomass to ethanol more economical.

Highly thermostable cellulase enzymes are secreted by the cellulolytic thermophile *Acidothermus cellulolyticus gen. nov., sp. nov. These are discussed in U.S. Patents 5,275,944 and 5,110,735*. This bacterium was originally isolated from decaying wood in an acidic, thermal pool at Yellowstone National Park and deposited with the American Type Culture Collection (ATCC) under collection number 43068 (Mohagheghi et al. 1986. Int. J. System. Bacteriol. 36:435-443).

The cellulase complex produced by this organism is known to contain several different cellulase enzymes with maximal activities at temperatures of 75°C to 83°C. These cellulases are resistant to inhibition from cellobiose, an end product of the reactions catalyzed by cellulose. Also, the cellulases from *Acidothermus cellulolyticus* are active over a broad pH range centered about pH 5, the pH at which yeasts are capable of fermenting cellobiose and glucose to ethanol. A high molecular weight cellulase isolated from growth broths of *Acidothermus cellulolyticus* was found to have a molecular weight of approximately 156,600 to 203,400 daltons by SDS-PAGE. This enzyme is described by U.S. patent #5,110,735.

A novel cellulase enzyme, known as the E1 endoglucanase, also secreted by *Acidothermus cellulolyticus* into the growth medium, is described in detail in U.S. Patent #5,275,944. This endoglucanase demonstrates a temperature optimum of 83°C and a specific activity of 40μmole glucose release from carboxymethylcellulose/min/mg protein. This E1 endoglucanase was further identified as having an isoelectric pH of

6.7 and a molecular weight of 81,000 daltons by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

It has been proposed to use recombinant cellulase enzymes to either augment or replace costly fungal enzymes for cellulose degradation (Lejeune, Colson, and Eveleigh, In Biosynthesis and Biodegradation of Cellulose, C. Haigler and P.J. Weimer, Eds., Marcel-Dekker, New York, NY 1991, pp. 623-672). The genes coding for *Acidothermus cellulolyticus* cellulases cloned into *Streptomyces lividans E. coli*, or other microbial host organisms could provide an abundant, inexpensive source of highly active enzymes. However, in order to produce recombinant E1 endoglucanase, the gene encoding this enzyme must be known and available.

Disclosure of Invention

It is an object of the present invention to clone the gene for the E1 endoglucanase from *Acidothermus cellulolyticus*.

It is another object of the present invention to transform and express this E1 endonuclease gene in a different microbial host under the same and/or a different gene regulatory system.

It is a further object of the present invention to prepare mutant E1 endoglucanases which have different properties from the natural enzyme.

It is another further object of the present invention to prepare hybrid endoglucanases, one portion of which corresponds to a portion of the sequence of the E1 endonuclease or its mutants.

It is yet another object of the present invention to hydrolyse cellulose in cellulosic materials by contacting the cellulosic material with the E1 endonuclease produced by expression of the gene.

The present invention describes the gene for and the nucleotide sequence of the segment of *Acidothermus cellulolyticus* DNA encoding the E1 endoglucanase gene. This 3004 base fragment of DNA is unique in nature and discretely defined. The natural gene contains the ribosome binding site, promotor, signal peptide, open reading frame, termination codon, a putative transcriptional terminator, and a transcriptional regulatory sequence which shows homology to sequences found upstream of cellulase genes isolated from other actinomycete bacteria.

The cloned gene may be expressed in other microorganisms under its natural promotor or another promotor recognized by the host microorganism. Alternatively, additional copies of the gene may be introduced into *Acidothermus cellulolyticus* to enhance expression of the enzyme. Additionally, DNA encoding one or more domains of the *Acidothermus cellulolyticus* E1 endoglucanase may be ligated to domains in compatible other endoglucanases to make a recombinant DNA capable of expressing a hybrid endoglucanase enzyme having beneficial properties from both endoglucanases.

Brief Description of Drawings

Figure 1 shows the 3004 base pair nucleotide sequence of the region of *Acidothermus cellulolyticus* genomic DNA which contains the E1 endoglucanase gene.

Figure 2 shows the amino acid translation of the coding sequence described in Figure 1.

Figure 3 shows a schematic illustration of the suspected domain architecture of the *Acidothermus cellulolyticus* E1 endoglucanase protein. This figure includes the general locations of the catalytic, linker, and cellulose binding domains aside an amino acid sequence map.

Figure 4 shows a schematic illustration of the putative transcriptional and translational regulatory sequences associated with the E1 endoglucanase gene aside a nucleotide sequence map.

Figure 5 shows the regions deleted for many deletion mutants and whether or not the protein product has endoglucanase activity.

Figure 6 shows an amino acid sequence comparison between the catalytic domains of *Bacillus polymyxa* ß-1,4-endoglucanase (GUN_BACPO), *Xanthomonas campestsis* ß-1,4-endoglucanase A (GUNA_XANPC_CAT), *Acidothermus cellulolyticus* E1 endoglucanase (E1 cat domain) and a consensus sequence.

Description of Preferred Embodiments

According to the present invention the gene for *Acidothermus cellulolyticus* E1 endoglucanase is cloned and expressed in a different microbial host. This enzyme is a β1-4 endoglucanase or endocellulase which can hydrolyze cellulose or xylan and is hereafter referred to as E1 endoglucanase. The result is a vastly improved rate of enzyme production, thereby lowering the cost of cellulase and the production of alcohol using cellulosic materials as substrates.

While endoglucanase alone is generally insufficient to completely hydrolyze cellulose, the enzyme product of the present invention may be used alone or preferably in combination with other cellulases to improve their effectiveness.

The coding portion of the gene appears to be 1686 base pairs long corresponding to 562 amino acids. The mature protein is only 521 amino acids long. Presumable the first 41 amino acids encode a signal sequence which is later cleaved from the E1 endoglucanase enzyme. The nucleotide and amino acid sequences may be seen in Figures 1 and 2 respectively. Review of the DNA sequence corresponding to the deduced amino acid sequence indicates that the gene contains an a gene architecture similar to other cellulase genes. The approximate gene architecture is shown in Figures 3 and 4.

The *Acidothermus cellulolyticus* E1 endoglucanase gene was cloned using standard recombinant DNA techniques as will be described below. Variations on these techniques are well known and may be used to reproduce the invention. Alternatively, the DNA molecule of the present invention can be produced through any of a variety of other means, preferably by application of recombinant DNA techniques or DNA synthesis of the gene. Techniques for synthesizing such molecules are disclosed by, for example, Wu et al, Prog. Nucl. Acid. Res. Molec. Biol. 21:101-141 (1978).

Standard reference works setting forth the general principles of recombinant DNA technology and cell biology include Watson et al., Molecular Biology of the Gene, Volumes I and II, Benjamin/Cummings Publishing Co., Inc., Menlo Park, CA (1987); Darnell et al., Molecular Cell Biology, Scientific American Books, Inc., New York, NY (1986); Lewin, Genes II, John Wiley & Sons, New York, NY (1985); Old et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2nd Ed., University of California Press, Berkeley, CA (1981); Sambrook et al, (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989)) and Albers et al., Molecular Biology of the Cell, 2nd Ed., Garland Publishing, Inc., New York, NY (1989).

Procedures for constructing recombinant molecules in accordance with the above-described method are disclosed by Sambrook et al., supra. Briefly, a DNA sequence encoding the endoglucanase gene of the present invention, or its functional derivatives, may be recombined with vector DNA in accordance with conventional

techniques, including blunt-ended or staggered-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, ligation with appropriate ligases. Part or all of the genes may be synthesized chemically in overlapping fragments which are hybridized together and annealed. The resulting vector may then be introduced into a host cell by transformation, transfection, electroporation, etc. Techniques for introducing a vector into a host cell are well known per se.

A vector is a DNA molecule, derived from a plasmid, bacteriophage or hybrid, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique restriction sites, and may be capable of autonomous replication or integration into the genome of a defined host or vehicle organism such that the cloned sequence is reproducible.

Another embodiment of the present invention relates specifically to the native 3004 nucleotide sequence of DNA encoding the *Acidothermus cellulolyticus* E1 endoglucanase enzyme and accompanying flanking sequences. This DNA encodes a 562 amino acid sequence which is shown in Figure 2. The molecular weight of the protein is believed to be about 60648 daltons. Other DNA sequences encoding the same 562 amino acids may readily be used as several amino acids are coded for by a plurality of different DNA triplet codons. Therefore, the gene encoding the *Acidothermus cellulolyticus* E1 endoglucanase may be any DNA which encodes that amino acid sequence.

One may also use an expression vector as the vector to clone the E1 endoglucanase gene. In such a situation, the host cell will also express the gene to produce the protein E1 endoglucanase. The protein may be separated, purified and assayed or assayed directly from the host cell or culture medium.

An expression vector is any autonomous element capable of replicating in a host cell independently of the host's chromosome, after a replicon has been incorporated into the autonomous element's genome. This vector has the ability to cause expression of the DNA inserted into it. Such DNA expression vectors include bacterial plasmids and phages and typically include promoter sequences to facilitate gene transcription.

In the situation where the E1 endoglucanase gene of the present invention has been cloned in a vector and expression has not occurred, the gene may be removed from the vector and inserted into an expression vector suitable for expressing the gene.

The DNA, is said to be capable of expressing a polypeptide if it contains nucleotide sequences which contain signals for transcriptional and translational initiation, and such sequences are operably linked to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the signals for transcriptional and translational initiation and the DNA sequence sought to be expressed are connected in such a way as to permit gene expression. The precise nature of the signals required for gene expression may from organism to organism.

The native promotor for *Acidothermus cellulolyticus* E1 endoglucanase may not be functional or efficient for the expression in certain microbial hosts. In such a situation, a suitable promotor region of DNA may be ligated upstream from the E1 endoglucanase gene to control its expression. In addition to the promotor, one may include regulatory sequences to either increase expression or to control expression. Expression may be controlled by an inducer or a repressor so that the recipient microorganism expresses the gene(s) only when desired.

A promoter or regulatory region contains a promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, CAAT sequence, and the like. Other sequences which regulate gene expression are considered regulatory sequences. In practice, the distinction may be blurred as the two regions may overlap each other. These sequences may be either the natural sequences from the *Acidothermus cellulolyticus* E1 endoglucanase gene, they may be from other genes, be synthetic or a combination of these.

If desired, the non-coding region 3' to the gene sequence coding for E1 endoglucanase may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence coding for the protein, the transcriptional termination signals may be

provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

For expressing the E1 endoglucanase gene, one may use a variety of microbial hosts including most bacteria, yeast, fungi and algae. Organisms which naturally produce a cellulase enzymes are preferred host cells along with easy to grow host cells and host cells known to express heterologous genes in large quantities.

If the host cell is a bacterium, generally a bacterial promoter and regulatory system will be used. For a typical bacterium such as E. coli., representative examples of well known promoters include trc, lac, tac, trp, bacteriophage lambda P_L, T7 RNA polymerase promoter, etc. When the expression system is yeast, examples of well known promoters include: GAL 1/GAL 10, alcohol dehydrogenase (ADH), his3, cycl, etc. For eukaryotic hosts, enhancers such as the yeast Ty enhancer, may be used.

Alternatively, if one wished for the E1 endoglucanase gene to be expressed at only a particular time, such as after the culture or host organism has reached maturity, an externally regulated promoter is particularly useful. Examples include those based upon the nutritional content of the medium (e.g. lac, trp, his), temperature regulation (e.g. temperature sensitive regulatory elements), heat shock promoters (e.g. HSP80A, U.S. Patent 5,187,267), stress response (e.g. plant EF1A promoter, U.S. Patent 5,177,011) and chemically inducible promoters (e.g. tetracycline inducible promoter or salicylate inducible promoter U.S. Patent 5,057,422).

Other suitable hosts for expressing E1 endoglucanase include *Bacillus*, *Xanthomonas*, *Trichoderma*, *Fusarium* and *Penicillium*, for example. These microorganisms also serve as sources of endoglucanase genes for the formation of mixed domain genes for the production of hybrid enzymes.

Expressing E1 endoglucanase in *E. coli* may be performed under control of a T7 bacteriophage promoter or other promotor functional in *E. coli*. Expression in *E. coli* has been enhanced by a factor of five relative to the native gene with the constructs of the present invention. Expression of the E1 endoglucanase coding sequence in *S. lividans* has been achieved under the control of the *tipA* promoter (thiostrepton-inducible) .

Intact native, variant or hybrid E1 endoglucanase proteins can be efficiently made in bacteria by providing a strong, promoter and an acceptable ribosome binding site. To express a prokaryotic gene that has an acceptable natural ribosome binding site, only a promoter must be supplied. Levels of expression may vary from less than 1% to more than 30% of total cell protein.

Chemical derivatives of the E1 endoglucanase DNA or the E1 endoglucanase protein are also included within the definition of that DNA or protein. Examples of chemical derivatives include but are not limited to: labels attached to the molecule, chemically linking the molecule to an additional substance, methylation, acylation, thiolation, chemical modification of a base or amino acid, etc.

The nucleotide sequence may be altered to optimize the sequence for a given host. Different organisms have different codon preferences as has been reported previously. Furthermore, the nucleotide sequence may be altered to provide the preferred three dimensional configuration of the mRNA produced to enhance ribosome binding and expression. Alternatively, the change can be made to enhance production of active enzyme, such as changing internal amino acids to permit cleavage of E1 endoglucanase from a fusion peptide or to add or subtract a site for various proteases. Oike, Y., et al., J. Biol. Chem. 257: 9751-9758 (1982); Liu, C., et al., Int. J. Pept. Protein Res. 21: 209-215 (1983). It should be noted that separation of E1 endoglucanase from a leader sequence is not necessary provided that the E1 endoglucanase activity is sufficiently acceptable.

Changes to the sequence such as insertions, deletions and site specific mutations can be made by random chemical or radiation induced mutagenesis, restriction endonuclease cleavage, transposon or viral insertion, oligonucleotide-directed site specific mutagenesis, or by such standard techniques as Botstein et al, Science 229: 193-210 (1985).

Such changes may be made in the present invention to alter the enzymatic activity, render the enzyme more susceptible or resistant to temperature or chemicals, alter regulation of the E1 endoglucanase gene, and to optimize the gene expression for any given host. These changes may be the result of either random changes or changes to a particular portion of the E1 endoglucanase molecule believed to be involved with a particular function. To further enhance expression, the final host organism may be

mutated so that it will change gene regulation or its production of the E1 endoglucanase gene product.

Such changes in either the nucleotide sequence or the amino acid sequences are considered variants of the natural sequences. Nucleotide sequence changes may be conservative and not alter the amino acid sequence. Such changes would be performed to change the gene expression or ability to easily manipulate the gene. Nucleotide sequence changes resulting in amino acid sequence differences are generally for altering the enzyme product to impart different biological properties, enhance expression or secretion or for ease in purification. Changes in the DNA sequence outside the coding region may also be made to enhance expression of the gene or to improve the ease of DNA manipulation.

The natural amino acid sequence is believed to contain a signal region and three domains corresponding as follows:

Key	From	To	Description
SIGNAL	1	41	Putative signal
SIGNAL	14	41	Putative signal (alternative)
DOMAIN	42	404	Catalytic domain
DOMAIN	405	458	Linker
DOMAIN	459	562	CBD

The N-terminal amino acid sequence determined corresponds to amino acids 42 to 79. For industrial uses, cellulase enzymes that display thermal stability, such as E1 endoglucanase, generally have enhanced stability under harsh process conditions as well as high temperatures. Since shear forces are applied during pumping and stirring, additional stability from this stress is desired. Other benefits include acid stability, a potential advantage with residual acid remaining from acid prehydrolysis of cellulosic materials and resistance to proteases which are produced by common contaminants.

While the term "variants" generally does not encompass large changes in the amino acid sequence, in the present application, the term "variants" includes a large number of changes outside the catalytic region of the endoglucanase. For example, a significant deletion of the native gene as described in Example 4 below. Other large

deletions outside the catalytic region such as in the signal, hinge, CBD domains or portions of the catalytic domain are also readily apparent and would be considered "variants".

For the purposes of this application, the terms "hybrid enzyme" or "hybrid protein" includes all proteins having at least one functional domain originating substantially from one protein and another functional domain substantially originating from at least one different protein. Signal sequences may be considered domains.

Hybrid enzymes of E1 endoglucanase may be prepared by ligating DNA encoding one or more E1 endoglucanase domains to one or more domains from a different cellulase gene. Representative examples of other cellulase genes which may be use are *Bacillus polymyxa* ß-1,4-endoglucanase (Baird et al, Journal of Bacteriology, 172: 1576-86 (1992)) and *Xanthomonas campestsis* ß-1,4-endoglucanase A (Gough et al, Gene 89: 53-59 (1990)). The number of domains in the hybrid protein may be the same or different from any natural enzyme. A large number of different combinations are possible.

It is further contemplated that one may include more than one catalytic domain in the hybrid enzyme. This may allow for increased specific activity. Also, a catalytic domain containing cellulase activity other than endonuclease activity may be included as well to reduce the number of cellulase enzymes one needs to add to a cellulosic substrate for polymer degradation.

Another preferred embodiment is to use the E1 endoglucanase produced by recombinant cells to hydrolyse cellulose in cellulosic materials for the production of sugars per se or for fermentation to alcohol. The processes for the fermentation of sugars to alcohol and its many variations are well known per se.

In situations where one wishes to simultaneously ferment the sugars produced by hydrolysis of cellulose, one may use yeast or Zymomonas as suitable hosts for introducing the E1 endoglucanase gene or use a mixed culture of an alcohol producing microbe and the E1 endoglucanase enzyme or microbe producing enzyme. If insufficient endoglucanase protein is released, the culture conditions may be changed to enhance release of enzyme. Other suitable hosts include any microorganism fermenting glucose to ethanol such as Lactobacillus or Clostridium and microorganisms fermenting a pentose to ethanol.

Either yeast or *Zymomonas* may be employed as a recombinant host for cellulase gene expression. However, yeast (*Saccharomyces cerevisiae*) is known to be a poor host for proteins when secretion into the medium is desired. The capacity of *Zymomonas* to secrete large amounts of proteins is not understood thoroughly of the present time. However, heterologous cellulase genes have been transferred into and expressed at fairly low levels in both *S. cerevisiae* (Bailey *et al.*, Biotechnol. Appl. Biochem. 17:65-76, (1993) and in *Zymomonas* (Su *et al.*, Biotech. Lett. 15:979-984, (1993).

Even if the genes for E1 endoglucanase are not secreted, considerable amounts of cell death and cell lysis occurs during processing due to shearing and pressure differences, thereby releasing some of the enzyme into the surrounding medium. Leakage of enzyme may be enhanced by a number of culture conditions which increase cell membrane permeability such as temperature and osmotic changes, surfactants, lytic agents (proteases, antibiotics, etc.) and physical stress.

Unless specifically defined otherwise, all technical or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

Example 1

Genome Library Construction, Library Screening, and Subcloning.

Genomic DNA was isolated from *Acidothermus cellulolyticus* and purified by banding on cesium chloride gradients. Genomic DNA was partially digested with Sau 3A and separated on agarose gels. DNA fragments in the range of 9-20 kilobase pairs were isolated from the gels. This purified Sau 3A digested genomic DNA was ligated into the Bam H1 acceptor site of purified EMBL3 lambda phage arms (Clontech, San Diego, CA). Phage DNA was packaged according to the manufacturer's specifications and plated on top of *E. coli* LE392 agar which contained the soluble cellulose analog, carboxymethylcellulose (CMC). The plates were incubated overnight (12-24 hours) to allow transfection and bacterial growth. Plates were stained with Congo Red followed by destaining with 1 M NaCl. Lambda plaques harboring endoglucanase clones showed up as unstained plaques on a red background.

Lambda clones which screened positive on CMC-Congo Red plates were purified by successive rounds of picking, plating and screening. Individual phage isolates were named SL-1, SL-2, SL-3 and SL-4. Subsequent subcloning efforts employed the SL-2 clone which contained an approximately 13.7 kb fragment of genomic DNA.

Standard methods for subcloning DNA fragments can be found in Molecular Cloning A Laboratory Manual (J. Sambrook, E.F. Fritsch and T. Maniatis, Cold Spring Harbor Laboratory Press, second edition, 1989). Purified SL-2 insert DNA was cut with BamH1, Pvu1 and EcoR1. Resulting fragments of DNA were individually purified by electrophoretic separation on agarose gels. BamH1 digestion yielded two fragments derived from gene SL-2 insert DNA, 2.3 and 9 kb in length. Pvu1 digestion yielded fragments of 0.7, 0.9, 1.7, 2.4, 3.3, and 3.7 kb. EcoR1 digestion produced insert-derived fragments of 0.2, 0.3, 1.9, 2.4 and 3.7 kb in length. Individual purified restriction fragments were ligated into plasmid vectors previously cut with the appropriate restriction enzyme. Specifically, the 2.3 and the 9 kb BamH1 fragments were ligated separately into BamH1 cut pBR322 and pGEM7. Pvu1 fragments were ligated separately into Pvu1 cut pBR322. The 3.7 kb Pvu1 fragment was also blunt ended by treatment with T4 DNA polymerase and ligated into the Sma1 site of pGEM7. EcoR1 fragments were ligated into EcoR1 cut pBR322.

Ligation products were transformed into competent *E. coli* DH5α cells and plated onto appropriate selective media (LB + 15 µg/ml tetracycline or LB + 50 µg/ml ampicillin) containing 1 mM of the substrate analog, 4-methylumbelliferyl-cellobioside (4-MUC), and grown overnight at 37°C. Cleavage of the 4-MUC by β-1,4-endoglucanase activity results in the formation of a highly fluorescent aglycone product, 4-methylumbelliferone. Plates were inspected for fluorescing colonies under long wave ultraviolet light to determine which subclones harbor fragments of *A. cellulolyticus* DNA encoding functional cellulase genes. Plasmids were purified from fluorescing colonies and the size of the subcloned DNA verified by restriction digestion. By these methods it was possible to determine that the 2.3 kb BamH1 fragment encodes a cellulase activity, as does the 3.7 kb Pvu1 fragment. It has been shown by Southern blot hybridization experiments that the 2.3 kb BamH1 fragment and the 3.7 kb Pvu1 fragment contain homologous DNA sequences. DNA sequencing

was performed with templates containing *A. cellulolyticus* DNA inserted into the plasmid pGEM7.

Subclone name	Description
p52	2.3 kb BamH1 fragment in BamH1 site of pGEM7
p53	2.3 kb BamH1 fragment in BamH1 site of pGEM7 (opposite orientation)
4-5	3.7 kb Pvu1 fragment in Sma1 site of pGEM7
4-9	3.7 kb Pvu1 fragment in Sma1 site of pGEM7 (opposite orientation)
9-1	1.4 kb EcoR1/Pvu1 fragment derived from 4-9 by EcoR1 cleavage and relegation

A 2.3 kb Bam H1 fragment and an overlapping 3.7 kb Pvu1 fragment were shown to express CMCase activity.

Bi-directional Deletion Subclones for Sequencing.

Bi-directional deletion subclones of the 2.3 kb Bam H1 subclone from SL-2 were produced using the commercially available Exo III/Mung bean nuclease deletion kit from Promega. A 2.3 kb BamH1 fragment isolated from clone SL-2 was cloned in both orientations into the BamH1 site of an *E. coli* vector called pGEM-7Zf(+) (Promega Corp., Madison, WI). These clones are referred to as p52 and p53, respectively. Two sets of nested deletion clones were produced according to the manufacturer's specifications using the Erase-a-Base® deletion system available from Promega. Deletions were constructed by double digesting the plasmid with HindIII and Kpn1. The 5' overhanging sequences resulting from HindIII cleavage provide a starting point for ExoIII deletion. The 3' overhanging sequences resulting from cleavage by Kpn1 protect the vector DNA from ExoIII digestion. Thus, deletions are unidirectional from the HindIII site, not bi-directional.

Double digested plasmid DNA was then exposed to digestion by the 3' to 5' exodeoxyribonuclease, ExoIII, and aliquots of the reaction were removed at various time points into a buffer which halts ExoIII activity. S1 nuclease, a single strand specific endonuclease, was then added to remove single stranded DNA and to blunt

end both ends of the deletion products. T4 DNA ligase was then used to re-circularize plasmid DNAs and the products were transformed into competent *E. coli* cells.

A representative sampling of the resulting clones are screened by restriction enzyme analysis of plasmid DNAs in order to estimate the extent of deletion. Deletions endpoints occurred fairly randomly along the sequence and clones were selected for sequencing such that deletion endpoints are spaced at approximately 100 to 300 bp intervals along the 2.3 kb BamH1 fragment. One set of clones is a succession of progressively longer deletions from one end of clone p52 and the other is a similar set of successively longer deletions from p53. Please refer to Figure 5 for the appropriate length of each deletion mutant. Each of the deletion clones was plated on MUC indicator plates to determine which still exhibited endoglucanase activity. Retention of β-1,4-glucanase activity in the deletion subclones is indicated by the symbol, "+"; lack of activity by the symbol, "-", after the name of each clone listed in Figure 5.

Manual DNA Sequencing.

Sequencing reactions were performed using double-stranded plasmid DNAs as templates. Templates used for DNA sequencing reactions included each of the plasmid DNAs diagrammed in Figure 5. In order to complete the sequencing of the E1 gene another subclone was employed as a template in conjunction with synthetic oligonucleotides used as primers. The 3.7 kb Pvu1 fragment from SL-2 was blunt ended with T4 DNA polymerase and cloned in both orientations into the Sma1 site of pGEM7, resulting in clones 4-5 and 4-9. The 3.7 kb Pvu1 fragment largely overlaps the 2.3 kb BamH1 subclone (as shown in Figure 5). Newly synthesized oligonucleotide primers were used to sequence the 810 base pairs downstream of the internal BamH1 located at position 2288 of the DNA sequence.

The reactions were carried out using alpha-^{35}S-dATP to label DNA synthesized using the T7 DNA polymerase kit provided by United States Biochemicals. Reaction products were separated on wedge acrylamide gels and were autoradiographed after fixation and drying. X-ray films were read using the gel reader apparatus (a model GP7 MarkII sonic digitizer, manufactured by Science Accessories Corp., Stratford, CT.) and GeneWorks™ software package provided by Intelligenetics, Inc. (Mountain View, CA). Sequences were checked and assembled using the same software package.

Example 2

Analysis of the Gene Coding for E1 Endoglucanase.

Three peptide sequences have been obtained from purified endoglucanase E1 from *Acidothermus cellulolyticus*. Thirty-eight amino acids have been determined from the N-terminus of the E1 protein by automated Edman degradation. The 38 amino acid sequence is identical to the previously determined (U.S. Patent #5,275,944) 24 N-terminal amino acids and extends that N-terminal sequence of the native protein by another 14 amino acids. The N-terminal sequences are as follows:

AGGGYWHTSG REILDANNVP VRIA (reported in U.S. #5,275,944)
AGGGYWHTSG REILDANNVP VRIAGINWFG FETXNYVV (this work)

A comparison of the translation of the nucleotide sequence data in Figure 1 and the peptide sequences available from purified E1 endoglucanase indicates that this clone encodes the E1 endoglucanase protein. The N-terminal 38 amino acid sequence is in exact agreement with the translation of the DNA sequence between nucleotides 947-1060 in Figure 1. This long sequence of 38 amino acids was not found in other entry in the Swiss-Prot database.

Example 3

Gene Architecture

While not wishing to be bound by any particular theory, the following hypothesis is presented. Figure 1 shows that the mature translation product beginning with a GCG codon at position 947-949 and extends to a TAA terminator codon at position 2410-2412. Since cellulases are secreted, presumably to gain access to their substrates, one may assume a signal peptide is present which assists in the secretion process *in vivo*. A nucleotide sequence apparently comprising the signal peptide for the E1 endoglucanase is encoded by the nucleotide sequence from 824-946. This stretch of 123 base pairs encodes 41 amino acids, beginning with a GTG (valine) codon. We postulate that the translation start site is the GTG codon at position 824-826 instead of the more usual ATG (methionine) codon (position 863-865) because of the proximity of the GTG start codon to a putative upstream ribosome binding sites (RBS), and because of the better amino-terminal charge density on the longer signal

peptide. Alternatively, the signal sequence may start with the methionine at position 14 of the apparent signal. For the purposes of gene manipulations, either signal sequence may be used.

The putative RBS for the E1 endoglucanase gene is pointed out by the excellent homology (8 of 9 residues) to the published 3' end of the *S. lividans* 16S rRNA at positions 772-779 (Bibb and Cohen, 1982, *Mol. Gen. Genet.* 187:265-77). Three direct repeats of a 10 bp sequence occur immediately downstream of the putative RBS sequence at positions 781-790, 795-804 and 810-817, and are boxed in Figure 1. Nucleotides 710-725 are underlined because they are homologous to the palindromic regulatory sequence first found by Cornell University which lies upstream of several cellulase genes isolated from *Thermomonospora fusca* (Lin and Wilson, 1988, *J. Bacteriol.* 170:3843-3846) and later in another Actinomycete bacterium, *Microbispora bispora* (Yablonsky et al. In Biochemistry & Genetics of Cellulose Degradation; Aubert, Beguin, Millet, Eds., Academic Press: New York, NY, 1988, pp 249-266).

Promoter sequences for the E1 endoglucanase gene are not readily defined. There is extreme diversity of promoter sequences in Streptomycete genes. However, it is believed that they probably reside between the putative upstream regulatory sequence (at 710-725) and the putative RBS (at 772-779). Regardless, the DNA sequence of Figure 1 contains the promotor. Nucleotides 2514-2560 are underlined because they comprise a nearly perfect dyad which may function as a transcriptional terminator, as has been observed for other Streptomycete genes (Molnar, In Recombinant Microbes for Industrial and Agricultural Applications, Murooka and Imanaka, Eds., Marcel-Dekker, New York, NY, 1994).

Figure 2 shows the putative signal sequence in lower case letters. An alternative signal sequence may begin at the methionine residue at position 14 in this sequence. The mature protein appears to begin at position 42. This has been confirmed by N-terminal amino acid sequencing of the purified native E1 endoglucanase protein from culture supernatants of *Acidothermus cellulolyticus* (boxed). The underlined sequence resembles the proline/serine/threonine-rich linker domain common to multi-domain microbial cellulases. The sequences following the linker domain appear to comprise the cellulose binding domain (CBD). This sequence

shows easily discernable, but not identical homology with CBD sequences from other cellulases. Sequences preceding the underlined linker domain appear to comprise the catalytic domain of the E1 endoglucanase. This catalytic domain sequence is similar to, but not identical to catalytic domain sequences from other bacterial cellulase proteins.

Example 4

Expression of Truncated E1 Endoglucanase

When the E1 endoglucanase gene is expressed in *E. coli* a product of the gene which has a lower molecular weight than the native gene product, or that which is expressed in *S. lividans* is detected. The native and *S. lividans* products run at 72 kDa on SDS polyacrylamide gels, whereas the *E. coli* product appears to run at approximately 60 kDa. Positive identification of the predominant gene products was performed by Western blotting techniques, using a monoclonal antibody specific for the E1 endoglucanase. This monoclonal antibody does not cross react with any other protein in *E. coli or A. cellulolyticus*. The purified *E. coli* product and the N-terminus of the polypeptide was sequenced by automated Edman degradation. The sequence is identical to that of the purified native E1 protein from *A. cellulolyticus*. Accordingly, the recombinant E1 gene product from *E. coli* is carboxy-terminally truncated by some mechanism in this host system.

Example 5

Modified E1 Endoglucanase Genes

The nucleotide sequence may be modified by random mutation or site specific mutation provided that the amino acid sequence is unchanged. In this manner, restriction endonuclease sites may be inserted or removed from the gene without altering the enzyme product. Additionally, certain host microorganisms are well known to prefer certain codons for enhanced expression. For example, Gouy et al, Nucleic Acids Research, 10(22): 7055-74 (1982). Any or all of the codons may be appropriately modified to enhanced expression. These changes constitute a conservative variant of the original DNA sequence.

Site specific mutation is a preferred method for inducing mutations in transcriptionally active genes (Kucherlapati, Prog. in Nucl. Acid Res. and Mol. Biol., 36:301 (1989)). This technique of homologous recombination was developed as a

method for introduction of specific mutations in a gene (Thomas et al., Cell, 44:419-428, 1986; Thomas and Capecchi, Cell, 51:503-512, 1987; Doetschman et al., Proc. Natl. Acad. Sci., 85:8583-8587, 1988) or to correct specific mutations within defective genes (Doetschman et al., Nature, 330:576-578, 1987).

The nucleotide sequence may also be modified in the same manner to produce changes in the amino acid sequence. Similar techniques may be used in the present invention to alter the amino acid sequence to change a protease or other cleavage site, enhance expression or to change the biological properties of the enzyme. Small deletions and insertions may also be used to change the sequence. These changes constitute a variant in the amino acid sequence.

This group of variants are those in which at least one amino acid residue in the peptide molecule has been removed and a different residue inserted in its place. For a detailed description of protein chemistry and structure, see Schulz, G.E. et al., Principles of Protein Structure, Springer-Verlag, New York, 1978, and Creighton, T.E., Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, 1983. The types of substitutions which may be made in the protein or peptide molecule of the present invention may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz et al. (supra) and Figure 3-9 of Creighton (supra). Base on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: ala, ser, thr (pro, gly);
2. Polar, negatively charged residues and their amides: asp, asn, glu, gln;
3. Polar, positively charged residues: his, arg, lys;
4. Large aliphatic, nonpolar residues: met, leu, ile, val (cys); and
5. Large aromatic residues: phe, tyr, trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking any side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation which is important in protein folding. Note the Schulz et al. would merge Groups 1 and 2, above. Note also that Tyr, because of its hydrogen bonding potential, has some kinship with Ser, Thr, etc. Substantial

changes in functional properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups, which will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (a) substitution of gly and/or pro by another amino acid or deletion or insertion of gly or pro; (b) substitution of a hydrophilic residue, e.g., ser or thr, for (or by) a hydrophobic residue, e.g., leu, ile, phe, val or ala; (c) substitution of a cys residue for (or by) any other residue; (d) substitution of a residue having an electro-positive side chain, e.g., lys, arg or his, for (or by) a residue having an electronegative charge, e.g., glu or asp; or (e) substitution of a residue having a bulky side chain, e.g., phe, for (or by) a residue not having such a side chain, e.g., gly.

Most deletions and insertions, and substitutions according to the present invention are those which do not produce radical changes in the characteristics of the protein or peptide molecule. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. For example, a variant typically is made by site-specific mutagenesis of the peptide molecule-encoding nucleic acid, expression of the variant nucleic acid in recombinant culture, and, optionally, purification from the culture, for example, by immunoaffinity chromatography using a specific antibody such as the monoclonal antibody used in Example 4, on a column (to absorb the variant by binding).

The activity of the microbial lysate or purified protein or peptide variant can be screened in a suitable screening assay for the desired characteristic. For example, the CMCase assay of Example 1 may be repeated with differing conditions to determine the enzyme activity under different conditions.

Modifications of such peptide properties as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, pH insensitivity, resistance to sheer stress, biological activity, expression yield, or the tendency to aggregate with carriers or into multimers are assayed by methods well known to the ordinarily skilled artisan.

Example 6

Mixed Domain E1 Endoglucanase Genes and Hybrid Enzymes

From the putative locations of the domains in the E1 endoglucanase gene given above and in Figure 3 and comparable cloned cellulase genes from other species, one can cleave between each domain and ligate it to one or more domains from a different gene. The similarity between all of the endoglucanase genes permit one to ligate one or more domains from the *Acidothermus cellulolyticus* E1 endoglucanase gene with one or more domains from an endoglucanase gene from one or more other microorganisms. Other representative endoglucanase genes include *Bacillus polymyxa* ß-1,4-endoglucanase (Baird et al, Journal of Bacteriology, 172: 1576-86 (1992)) and *Xanthomonas campestsis* ß-1,4-endoglucanase A (Gough et al, Gene 89: 53-59 (1990)). The result of the fusion of the two domains will, upon expression, be a hybrid enzyme. For ease of manipulation, restriction enzyme sites may be previously added to the respective genes by site-specific mutagenesis. If one is not using one domain of a particular gene, any number of any type of change including complete deletion may be made in the unused domain for convenience of manipulation.

The foregoing description of the specific embodiments reveal the general nature of the invention so that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

All references mentioned in this application are incorporated by reference.

Claims

1. A DNA comprising a DNA encoding the following amino acid sequence:

AGGGYWHTSGREILDANNVPVRIAGINWFGFETCNYVVHGLWSRDYRSMLD
QIKSLGYNTIRLPYSDDILKPGTMPNSINFYQMNQDLQGLTSLQVMDKIVAYA
GQIGLRIILDRHRPDCSGQSALWYTSSVSEATWISDLQALAQRYKGNPTVVGF
DLHNEPHDPACWGCGDPSIDWRLAAERAGNAVLSVNPNLLIFVEGVQSYNGD
SYWWGGNLQGAGQYPVVLNVPNRLVYSAHDYATSVYPQTWFSDPTFPNNM
PGIWNKNWGYLFNQNIAPVWLGEFGTTLQSTTDQTWLKTLVQYLRPTAQYG
ADSFQWTFWSWNPDSGDTGGILKDDWQTVDTVKDGYLAPIKSSIFDPVGASA
SPSSQPSPSVSPSPSPSPSASRTPTPTPTPTASPTPTLTPTATPTPTASPTPSPTAA
SGARCTASYQVNSDWGNGFTVTVAVTNSGSVATKTWTVSWTFGGNQTITNS
WNAAVTQNGQSVTARNMSYNNVIQPGQNTTFGFQASYTGSNAAPTVACAAS

or variants thereof.

2. The DNA according to claim 1 further comprising the following sequence attached to an amino terminal end:

MLRVGVVVAVLALVAALANLAVPRPARA

or variants thereof.

3. The DNA according to claim 2 further comprising the following sequence attached to an amino terminal end:

VPRALRRVPGSRV or variants thereof.

4. The DNA according to claim 1 comprising the following sequence:

GGATCCACGT TGTACAAGGT CACCTGTCCG TCGTTCTGGT AGAGCGGCGG 50
GATGGTCACC CGCACGATCT CTCCTTTGTT GATGTCGACG GTCACGTGGT 100
TACGGTTTGC CTCGGCCGCG ATTTTCGCGC TCGGGCTTGC TCCGGCTGTC 150
GGGTTCGGTT TGGCGTGGTG TGCGGAGCAC GCCGAGGCGA TCCCAATGA 200
GGCAAGGGCA AGAGCGGAGC CGATGGCACG TCGGGTGGCC GATGGGGTAC 250
GCCGATGGGG CGTGGCGTCC CCGCCGCGGA CAGAACCGGA TGCGGAATAG 300
GTCACGGTGC GACATGTTGC CGTACCGCGG ACCCGGATGA CAAGGGTGGG 350
TGCGCGGGTC GCCTGTGAGC TGCCGGCTGG CGTCTGGATC ATGGGAACGA 400
TCCCACCATT CCCCGCAATC GACGCGATCG GGAGCAGGGC GGCGCGAGCC 450
GGACCGTGTG GTCGAGCCGG ACGATTCGCC CATACGGTGC TGCAATGCCC 500

AGCGCCATGT TGTCAATCCG CCAAATGCAG CAATGCACAC ATGGACAGGG 550
ATTGTGACTC TGAGTAATGA TTGGATTGCC TTCTTGCCGC CTACGCGTTA 600
CGCAGAGTAG GCGACTGTAT GCGGTAGGTT GGCGCTCCAG CCGTGGGCTG 650
GACATGCCTG CTGCGAACTC TTGACACGTC TGGTTGAACG CGCAATACTC 700
CCAACACCGA TGGGATCGTT CCCATAAGTT TCCGTCTCAC AACAGAATCG 750
GTGCGCCCTC ATGATCAACG TGAAAGGAGT ACGGGGGAGA ACAGACGGGG 800
GAGAAACCAA CGGGGGATTG GCGGTGCCGC GCGCATTGCG GCGAGTGCCT 850
GGCTCGCGGG TGATGCTGCG GGTCGGCGTC GTCGTCGCGG TGCTGGCATT 900
GGTTGCCGCA CTCGCCAACC TAGCCGTGCC GCGGCCGGCT CGCGCCGCGG 950
GCGGCGGCTA TTGGCACACG AGCGGCCGGG AGATCCTGGA CGCGAACAAC 1000
GTGCCGGTAC GGATCGCCGG CATCAACTGG TTTGGGTTCG AAACCTGCAA 1050
TTACGTCGTG CACGGTCTCT GGTCACGCGA CTACCGCAGC ATGCTCGACC 1100
AGATAAAGTC GCTCGGCTAC AACACAATCC GGCTGCCGTA CTCTGACGAC 1150
ATTCTCAAGC CGGGCACCAT GCCGAACAGC ATCAATTTTT ACCAGATGAA 1200
TCAGGACCTG CAGGGTCTGA CGTCCTTGCA GGTCATGGAC AAAATCGTCG 1250
CGTACGCCGG TCAGATCGGC CTGCGCATCA TTCTTGACCG CCACCGACCG 1300
GATTGCAGCG GGCAGTCGGC GCTGTGGTAC ACGAGCAGCG TCTCGGAGGC 1350
TACGTGGATT TCCGACCTGC AAGCGCTGGC GCAGCGCTAC AAGGGAAACC 1400
CGACGGTCGT CGGCTTTGAC TTGCACAACG AGCCGCATGA CCCGGCCTGC 1450
TGGGGCTGCG GCGATCCGAG CATCGACTGG CGATTGGCCG CCGAGCGGGC 1500
CGGAAACGCC GTGCTCTCGG TGAATCCGAA CCTGCTCATT TTCGTCGAAG 1550
GTGTGCAGAG CTACAACGGA GACTCCTACT GGTGGGGCGG CAACCTGCAA 1600
GGAGCCGGCC AGTACCCGGT CGTGCTGAAC GTGCCGAACC GCCTGGTGTA 1650
CTCGGCGCAC GACTACGCGA CGAGCGTCTA CCCGCAGACG TGGTTCAGCG 1700
ATCCGACCTT CCCCAACAAC ATGCCCGGCA TCTGGAACAA GAACTGGGGA 1750
TACCTCTTCA ATCAGAACAT TGCACCGGTA TGGCTGGGCG AATTCGGTAC 1800
GACACTGCAA TCCACGACCG ACCAGACGTG GCTGAAGACG CTCGTCCAGT 1850
ACCTACGGCC GACCGCGCAA TACGGTGCGG ACAGCTTCCA GTGGACCTTC 1900
TGGTCCTGGA ACCCCGATTC CGGCGACACA GGAGGAATTC TCAAGGATGA 1950
CTGGCAGACG GTCGACACAG TAAAAGACGG CTATCTCGCG CCGATCAAGT 2000
CGTCGATTTT CGATCCTGTC GGCGCGTCTG CATCGCCTAG CAGTCAACCG 2050
TCCCCGTCGG TGTCGCCGTC TCCGTCGCCG AGCCCGTCGG CGAGTCGGAC 2100
GCCGACGCCT ACTCCGACGC CGACAGCCAG CCCGACGCCA ACGCTGACCC 2150
CTACTGCTAC GCCCACGCCC ACGGCAAGCC CGACGCCGTC ACCGACGGCA 2200
GCCTCCGGAG CCCGCTGCAC CGCGAGTTAC CAGGTCAACA GCGATTGGGG 2250

CAATGGCTTC ACGGTAACGG TGGCCGTGAC AAATTCCGGA TCCGTCGCGA 2300
CCAAGACATG GACGGTCAGT TGGACATTCG GCGGAAATCA GACGATTACC 2350
AATTCGTGGA ATGCAGCGGT CACGCAGAAC GGTCAGTCGG TAACGGCTCG 2400
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GCAGCAAGTT AATACGTCGG GGAGCCGACG GGAGGGTCCG GACCGTCGGT 2550
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GGTACCAGAG AGGAACGACA CGAATGCCCG CCATCTCAAA ACGGCTGCGA 2650
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GGCGATGCAG CATCCTGCCA TCGCCGCGAC GCACGTCGAC AATCCCTATG 2750
CGGGAGCGAC CTTCTTCGTC AACCCGTACT GGGCGCAAGA AGTACAGAGC 2800
GAACGGCGAA CCAGACCAAT GCCACTCTCG CAGCGAAAAT GCGCGTCGTT 2850
TCCACATATT CGACGGCCGT CTGGATGGAC CGCATCGCTG CGATCAACGG 2900
CGTCAACGGC GGACCCGGCT TGACGACATA TCTGGACGCC GCCCTCTCCC 2950
AGCAGCAGGG AACCACCCCT GAAGTCATTG AGATTGTCAT CTACGATCTG 3000
CCGG 3004

or fragments thereof.

5. A vector comprising the DNA according to claim 1 and a vector sequence encoding either an origin of replication or an integration site for a host genome.

6. A vector according to claim 5 further comprising DNA encoding a signal sequence operably linked thereto.

7. A vector according to claim 5 further comprising exogenous regulatory sequences capable of causing expression of said DNA in a suitable host.

8. A recombinant microorganism containing the vector according to claim 5.

9. A recombinant microorganism containing the vector according to claim 6.

10. A recombinant microorganism containing the vector according to claim 7.

11. A recombinant microorganism according to claim 5 wherein a genus of said microorganism is selected from the group consisting of *Saccharomyces*, *Zymomonas* and *Escherichia*.

12. A method for producing an endoglucanase comprising culturing the recombinant microorganism according to claim 8 in a vessel under culture conditions sufficient to express said DNA and recovering said endoglucanase therefrom.

13. The method according to claim 12, further comprising separating the recombinant microorganism from microbial medium and recovering said endoglucanase from the medium.

14. A method for producing an endoglucanase according to claim 12, further comprising effectively increasing the permeability of a membrane of the recombinant microorganism to permit release of said endoglucanase.

15. A method of hydrolyzing cellulose in a cellulosic substrate comprising, mixing the microorganism of claim 8, or an extract thereof, with the cellulosic substrate, and incubating under conditions permitting cellulose hydrolysis by an endoglucanse.

16. A method of hydrolyzing cellulose in a cellulosic substrate comprising, mixing the medium containing endoglucanase of claim 13 with the cellulosic substrate, and incubative under conditions permitting cellulose hydrolysis by an endoglucanse.

17. An endoglucanase containing extract of the microorganism of claim 8.

18. A method for producing alcohol comprising fermenting hydrolyzed cellulose produced by the process of claim 15 and recovering the alcohol produced therefrom.

19. A method for producing alcohol comprising fermenting hydrolyzed cellulose produced by the process of claim 16 and recovering the alcohol produced therefrom.

20. A carboxy terminally truncated E1 endoglucanase which has an apparent molecular weight of approximately 60 kDa by SDS-PAGE, capable of being produced by expression of the DNA of claim 1 in *E. coli*.

21. A DNA comprising at least one domain but not all of the domains of E1 endoglucanase or a variant thereof.

22. The DNA according to claim 21 further comprising at least one domain from a cellulase gene other than E1 endoglucanase.

23. The DNA according to claim 22 wherein the DNA encodes a protein having a cellulase activity.

24. The DNA according to claim 23 wherein the cellulase activity is an endoglucanase activity.

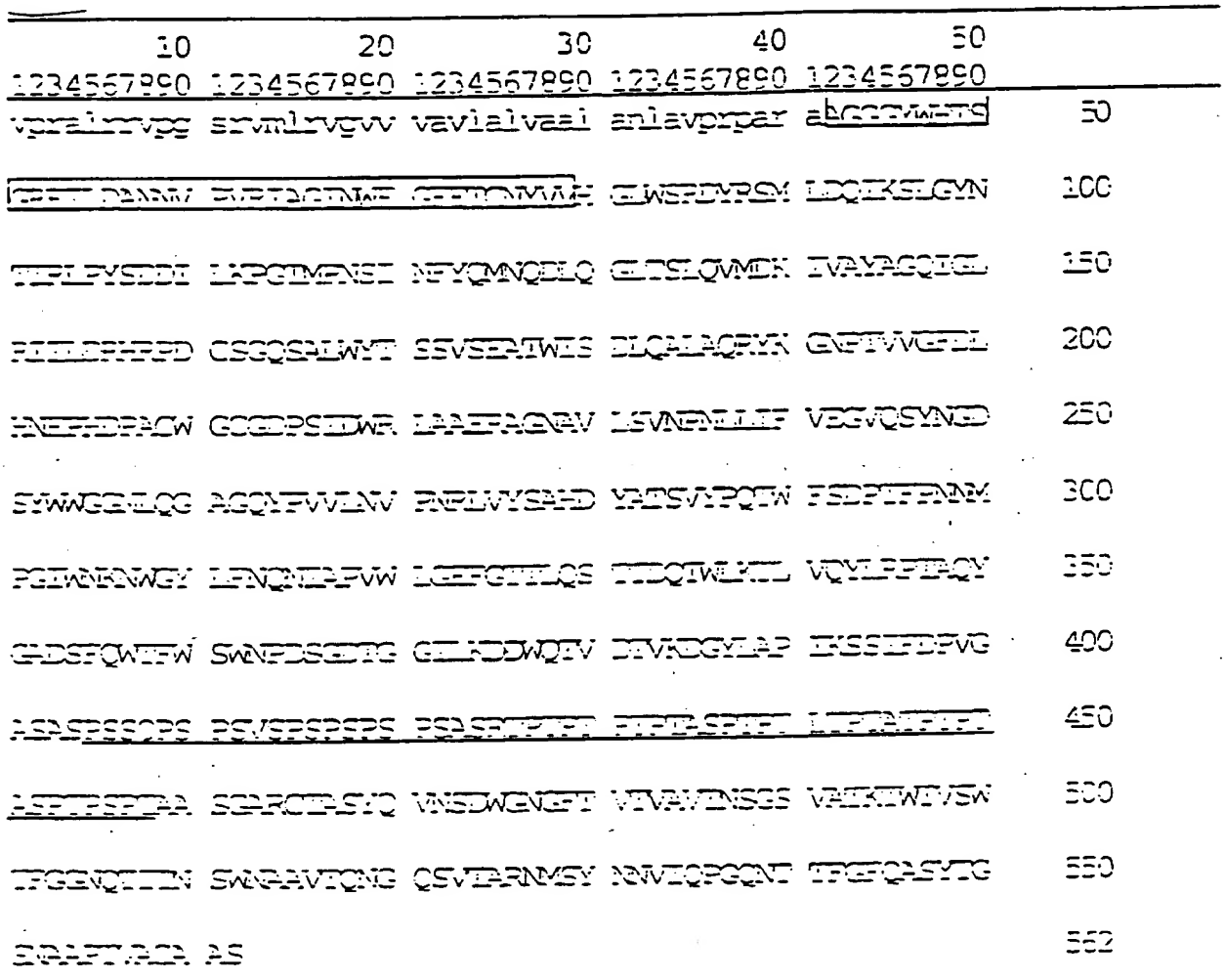
25. A hybrid protein having an amino acid sequence encoded by the DNA of claim 22.

26. A hybrid protein having an amino acid sequence encoded by the DNA of claim 23.

27. A hybrid protein having an amino acid sequence encoded by the DNA of claim 24.

FIGURE 1

FIGURE 2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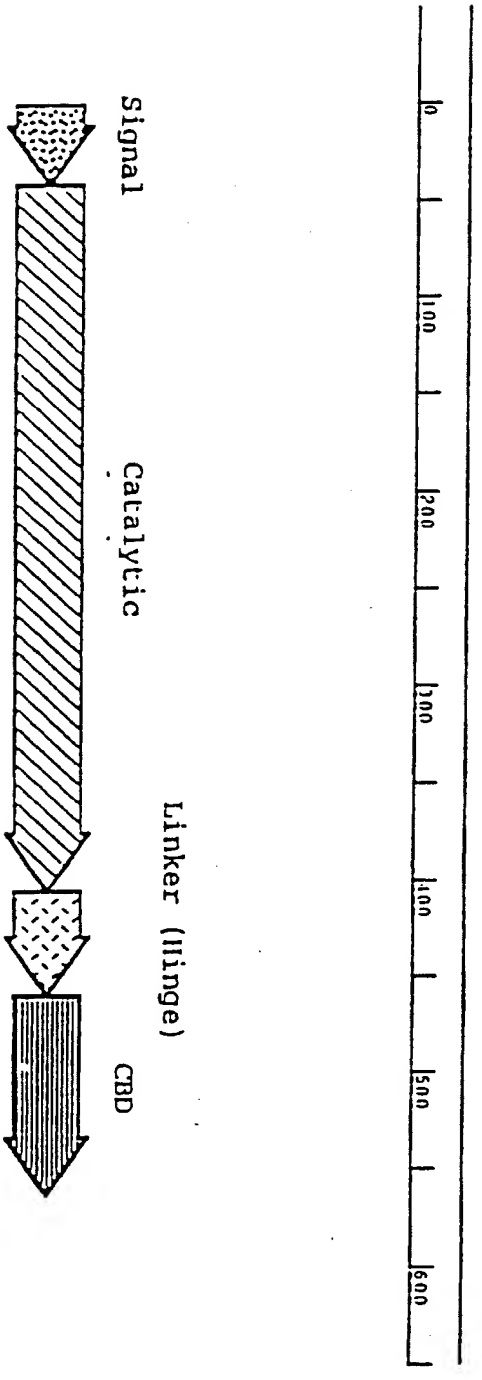


FIGURE 3

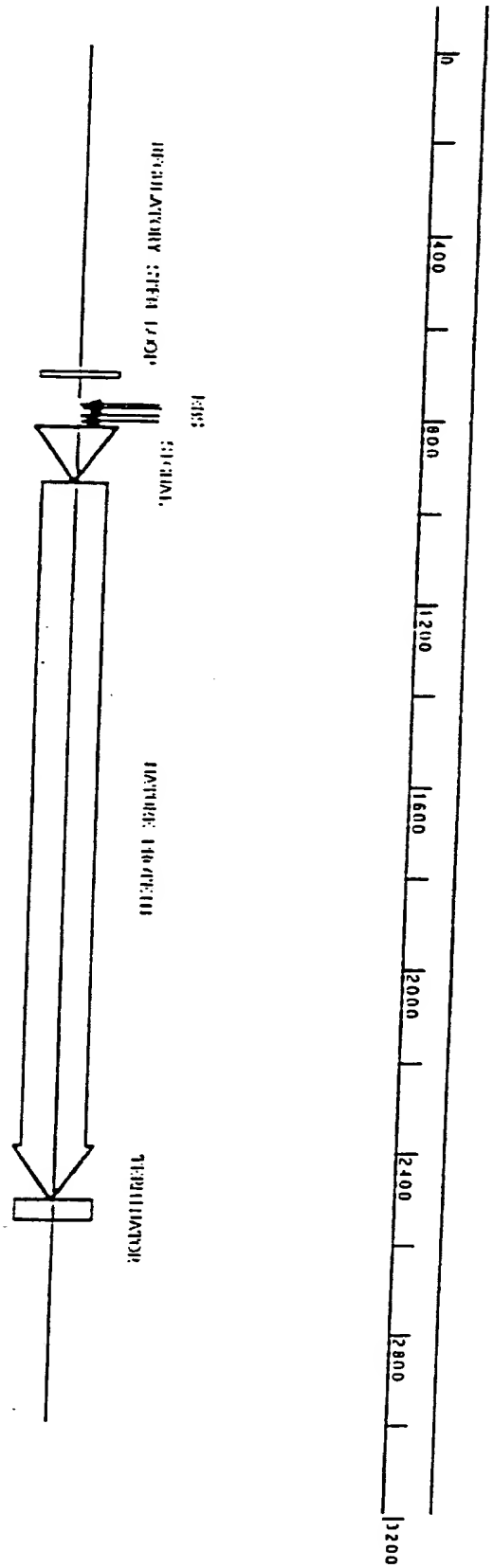


FIGURE 4

FIGURE 5

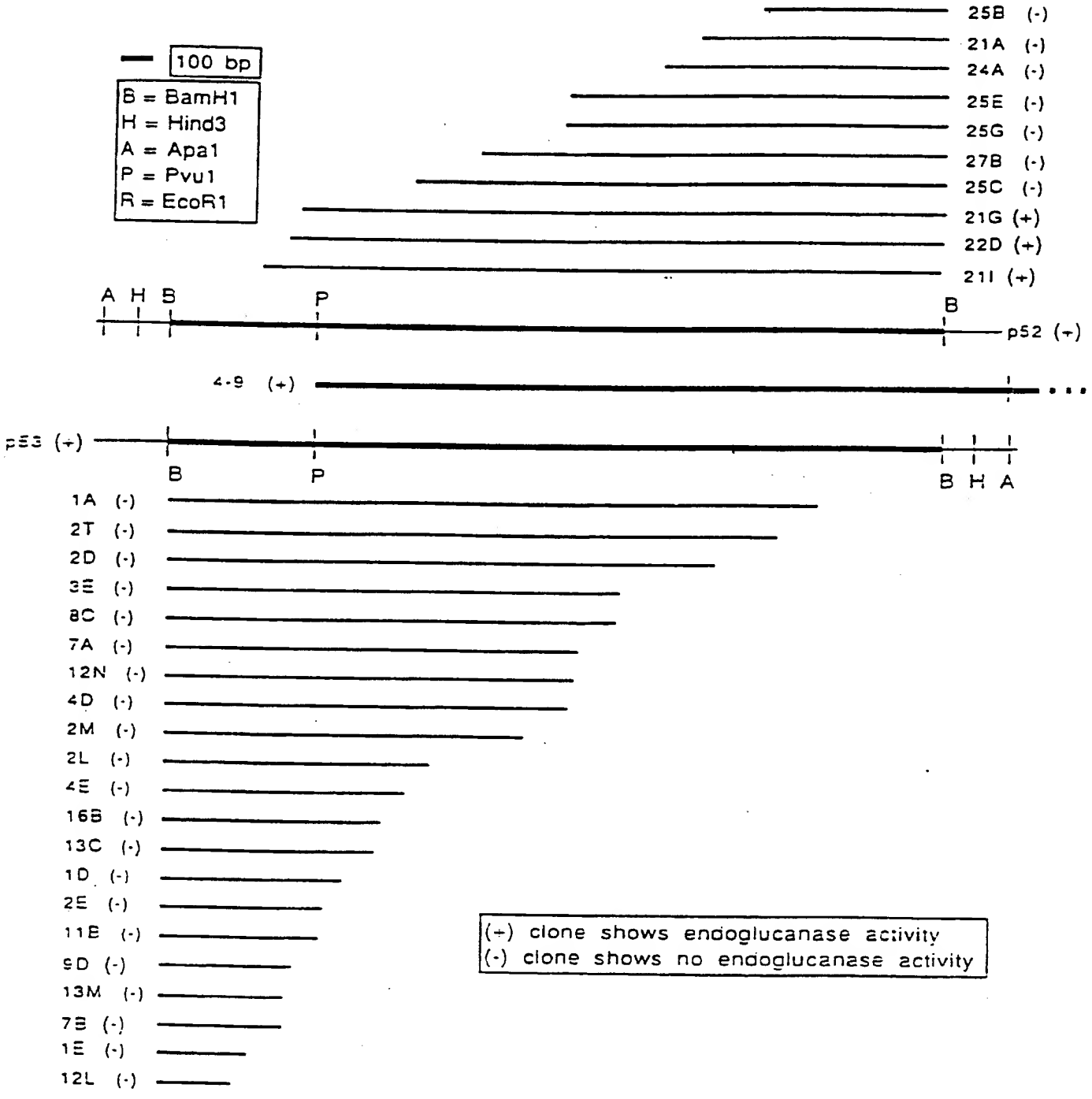
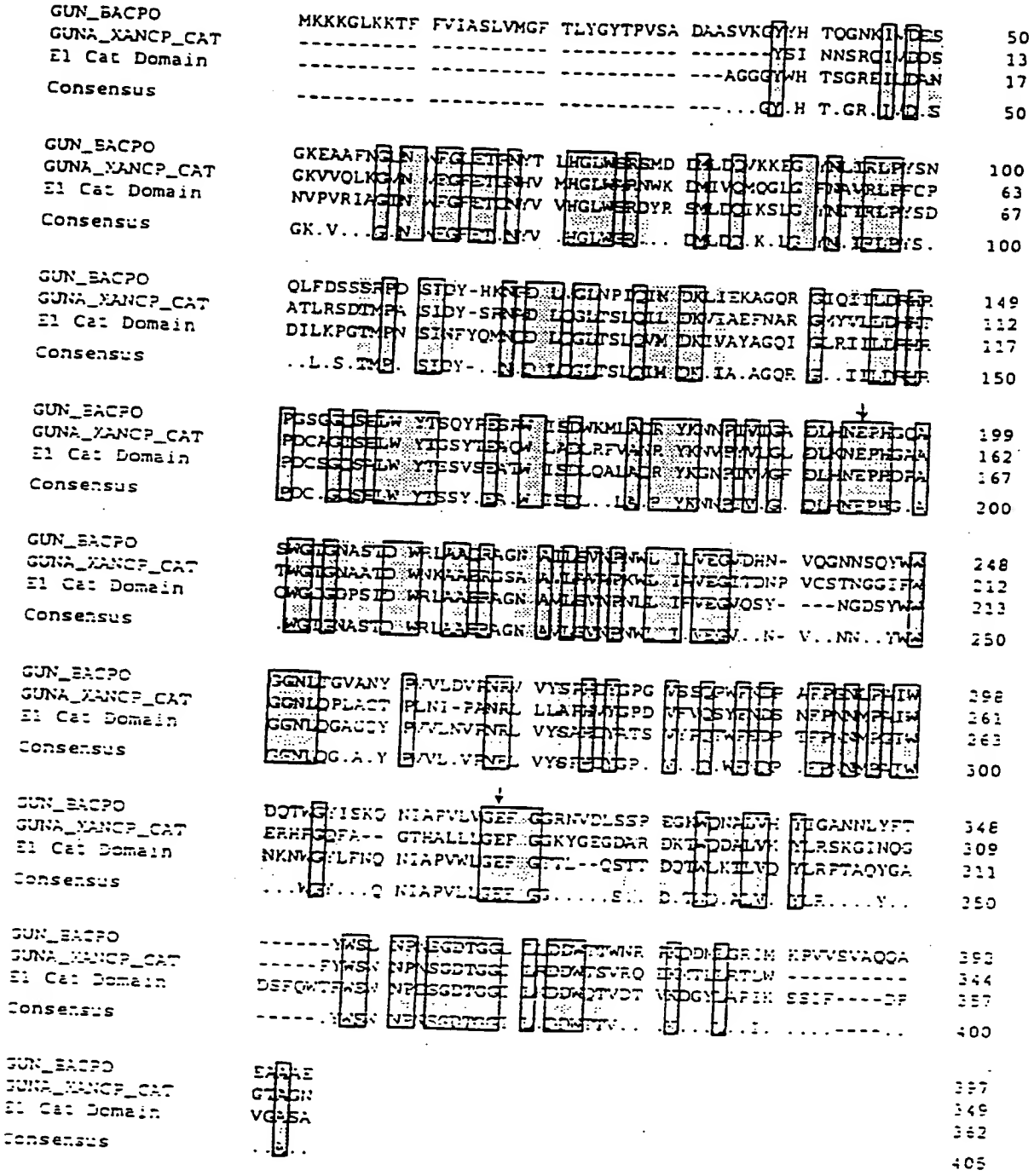


FIGURE 6



INTERNATIONAL SEARCH REPORT

Inte...tional application No.
PCT/US95/08868

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/69.1, 209, 252.3, 320.1; 536/22.1, 23.1, 23.2, 23.4, 23.7
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 209, 252.3, 320.1; 536/22.1, 23.1, 23.2, 23.4, 23.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, CA
search terms: acidothermus cellulolyticus, cellulolyticus, cellulase?, endoglucanase, beta 1-4 endoglucanase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 5,275,944 (HIMMEL ET AL) 04 January 1994, see entire document.	1-27

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search 17 SEPTEMBER 1995	Date of mailing of the international search report 26 OCT 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Hyosuk Kim Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

In. .ational application No.
PCT/US95/08868

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C07H 19/00, 21/00, 21/02, 21/04; C12N 1/20, 9/42, 15/00, 15/09, 15/63, 15/70, 15/74; C12P 21/06

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